Biochemical characterization of recombinant polypeptides corresponding to the predicted βαα fold in Aux/IAA proteins

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Abstract The plant hormone indoleacetic acid (IAA or auxin) transcriptionally activates a select set of early genes. The *Aux/IAA* class of early auxin-responsive genes encodes a large family of short-lived, nuclear proteins. Aux/IAA polypeptides homo- and heterodimerize, and interact with auxin-response transcription factors (ARFs) via C-terminal regions conserved in both protein families. This shared region contains a predicted βαα motif similar to the prokaryotic β-ribbon DNA binding domain, which mediates both protein dimerization and DNA recognition. Here, we show by circular dichroism spectroscopy and by chemical cross-linking experiments that recombinant peptides corresponding to the predicted βαα region of three Aux/IAA proteins from *Arabidopsis thaliana* contain substantial α-helical secondary structure and undergo homo- and heterotypic interactions in vitro. Our results indicate a similar biochemical function of the plant βαα domain and suggest that the βαα fold plays an important role in mediating combinatorial interactions of Aux/IAA and ARF proteins to specifically regulate secondary gene expression in response to auxin.

Key words: Auxin; Aux/IAA protein; βαα domain; Protein cross-linking; Secondary structure analysis; *Arabidopsis thaliana*

1. Introduction

The plant hormone auxin, typified by the naturally prevalent indole-3-acetic acid, is a key regulator of plant growth and development [1,2]. Auxin transcriptionally activates a select set of immediate-early genes that are thought to mediate the various effects of auxin in plants [3,4]. Three different classes of auxin-specific, early response genes have been characterized in various species and are known as the *SAUR*, *GH3*, and *Aux/IAA* genes (review in [5]). Functional promoter analyses of members of each auxin-specific gene class have revealed conserved AuxREs (review in [6,7]). The use of highly active, synthetic AuxREs has led to the recent discovery of ARF-1 and related transcription factors that bind to AuxREs conserved in many early auxin-inducible genes, including members of the *Aux/IAA* class [8–10].

With the exception of the *Aux/IAA* class, however, little is known about the proteins encoded by early auxin genes and about their functions in auxin action. As indicated by the isolation of at least 20 *IAA* genes in *Arabidopsis thaliana*, large multigene families encode Aux/IAA proteins in plants [11,12]. Biochemical, molecular, and genetic studies suggest that Aux/IAA polypeptides play a central role in auxin signaling and plant development. *Aux/IAA* genes encode short-lived nuclear proteins that homo- and heterodimerize and that share four islands of amino acid sequence conservation, designated conserved domains I–IV [11–13] (see Fig. 1A). Semidominant missense mutations in two *Aux/IAA* genes of *A. thaliana*, *IAA3* and *IAA17*, alter invariant amino acid residues in conserved domain II and are responsible for the pleiotropic morphological phenotype of *shy2* and *axr3* plants, respectively [14,15]. Intragenic suppressor mutations have been identified in *AXR3/IAA17* that largely revert the *axr3* phenotype to wild type appearance [15]. Intriguingly, the second-site mutations affect conserved domains I, III, and IV of IAA17, suggesting that all four conserved domains are important for Aux/IAA protein function.

A conspicuous structural feature of Aux/IAA proteins is centered on conserved domain III. This region is predicted to adopt an amphipathic βαα fold that is significantly similar to the βαα fold of prokaryotic transcriptional repressors such as Arc and MetJ [13]. The prokaryotic βαα fold mediates both dimerization of Arc monomers and DNA recognition of operator half-sites by Arc dimers [16]. Based on the predicted βαα motif, we have proposed that Aux/IAA proteins are transcriptional regulators of secondary gene expression in response to auxin [13]. Primary structure elements conserved in Aux/IAA and ARF proteins further support this hypothesis. Interestingly, the C-terminal regions of most ARF proteins contain Aux/IAA-like conserved domains III and IV, which compose the C-terminus of Aux/IAA proteins [9,10]. These C-terminal regions mediate both intra- and interfamily protein-protein interactions of Aux/IAA and ARF family members [9,12]. Therefore, the predicted amphipathic βαα fold of conserved domain III in Aux/IAA and ARF proteins likely plays an important role in mediating such homo- and heterotypic interactions. To date, there is no direct biochemical evidence to indicate that the predicted βαα motif of domain III is a folded protein domain and that this region mediates dimerization. To probe the secondary structure of the predicted βαα fold and to test its proposed involvement in protein-protein interactions, we have expressed and purified several recombinant peptides derived from conserved domain III of Aux/IAA proteins. Here, we show by circular dichroism analysis and by cross-linking studies that the predicted βαα fold of Aux/IAA proteins is indeed a folded protein domain and dimerizes in vitro.

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Abbreviations: ARF, auxin response factor; AuxRE, auxin responsive *cis* element; BS^3, bi(sulfosuccinimidyl) suberate; CD, circular dichroism; IAA, indoleacetic acid; Ni-NTA, Ni-nitrilotriacetic acid; SAUR, small auxin-upregulated mRNAs

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2. Materials and methods

2.1. Construction of plasmids

Partial cDNA fragments encoding the predicted βαα domain of *A. thaliana* proteins IAA1 (aa 63–122), IAA2 (aa 68–122), and IAA3 (aa 80–133) [11] were generated by PCR and subcloned into pQE plasmids (Qiagen) to produce recombinant peptides with C-terminal $(His)_6$ tags. The PCR products were ligated with the *SphI/BglI* 5′-acceptor fragment of pQE-7, which provides the translational initiation codon, and with the *BglII/BglI* 3′-acceptor fragment of pQE-16, which provides the $(His)_6$ tag. All subcloned cDNA fragments were verified by DNA sequencing.

2.2. Expression and purification of $(His)_6$-tagged βαα peptides

The recombinant βαα peptides were expressed in *Escherichia coli* M15[pREP4] (Qiagen) at 37°C for 4 h by induction with 1 mM isopropyl β-D-thiogalactopyranoside. The bacterial pellets derived from 0.5 l cultures were extracted at room temperature for 1 h by stirring in 12.5 ml buffer A (6 M guanidinium hydrochloride, 100 mM Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). The extracts were cleared by centrifugation at 20 000×*g* for 30 min, and the supernatants were applied to a column containing 1 ml bed volume of Ni-NTA agarose (Qiagen) equilibrated in buffer A. The columns were subsequently washed with 10 ml each of buffer A and buffer B (8 M urea, 100 mM Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). A final wash with buffer C (buffer B, pH 6.3) was conducted until the absorbance of the flow-through at 280 nm was less than 0.01. Recombinant $(His)_6$-tagged peptides were eluted with buffer D (buffer B, pH 5.9) in 1 ml aliquots. Each fraction was tested by SDS-PAGE. Appropriate fractions were pooled, and the βαα peptides were renatured by dialyzing overnight against 10 mM K_2HPO_4/KH_2PO_4, pH 8.0, 100 mM KCl, 5 mM EDTA.

2.3. Protein determination

To determine accurately the concentrations of peptide stock solutions, protein concentrations were determined by measuring tyrosine absorbance in 6 M guanidinium hydrochloride, 20 mM K_2HPO_4/KH_2PO_4, pH 6.5 [17]. The following extinction coefficients were used: 7250 M^{-1} cm^{-1} for βαα-1 and βαα-2, which contain five tyrosine residues each; and 5800 M^{-1} cm^{-1} for βαα-3, which contains four tyrosine residues.

2.4. Protein cross-linking and SDS-PAGE

For protein cross-linking experiments, reactions were carried out at 25°C in a total volume of 50 μl containing 50 μg/ml Ni-NTA purified $(His)_6$-tagged βαα peptides or ribonuclease A, 10 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, 100 mM NaCl and 0.5 mM BS^3 (Pierce). Control reactions received no BS^3. Reactions were terminated after 1 min by the addition of 50 μl 2×SDS sample buffer and boiling for 5 min. SDS-PAGE on 17% acrylamide gels and Coomassie staining of the gels were performed according to [18].

2.5. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco J-720 spectropolarimeter. Far-UV CD spectra of renatured, Ni-NTA purified $(His)_6$-tagged βαα peptides were recorded at 20°C from 300 nm to 190 nm in 10 mM K_2HPO_4/KH_2PO_4, pH 8.0, 100 mM KCl at a peptide concentration of 50 μg/ml. The observed CD signals were converted to the normalized molar ellipticity [19], and the CD spectra were analyzed by the method of Greenfield and Fasman [20].

3. Results

3.1. Expression and purification of recombinant domain III polypeptides

To probe by biochemical means the predicted βαα fold of Aux/IAA proteins, we expressed in *E. coli* three recombinant polypeptides, hereafter termed βαα-1, βαα-2, and βαα-3, which correspond to conserved domain III of *A. thaliana* proteins IAA1, IAA2, and IAA3, respectively (see Fig. 1B). All βαα peptides were expressed as C-terminal $(His)_6$-tagged fusion proteins to facilitate their purification from bacterial extracts. Analysis of the solubility of the bacterially expressed βαα peptides indicated that they form inclusion bodies in *E. coli* under various expression conditions tested. Insolubility of the recombinant βαα peptides precluded their purification under native conditions and necessitated the use of denaturing agents for purification by affinity chromatography. As shown in Fig. 2, sufficiently pure preparations of recombinant, denatured βαα-1, βαα-2, and βαα-3 peptides were obtained, with yields of 5–15 mg/l of culture. The apparent molecular mass observed for βαα-1 and βαα-2 closely matches the calculated value of 8.0 kDa and 7.5 kDa, respectively, whereas the apparent molecular mass of the βαα-3 peptide is slightly higher (7.6 kDa) than its expected value of 7.2 kDa (Fig. 2, compare lanes 4, 6, and 8). Minor protein bands of an apparent mo-

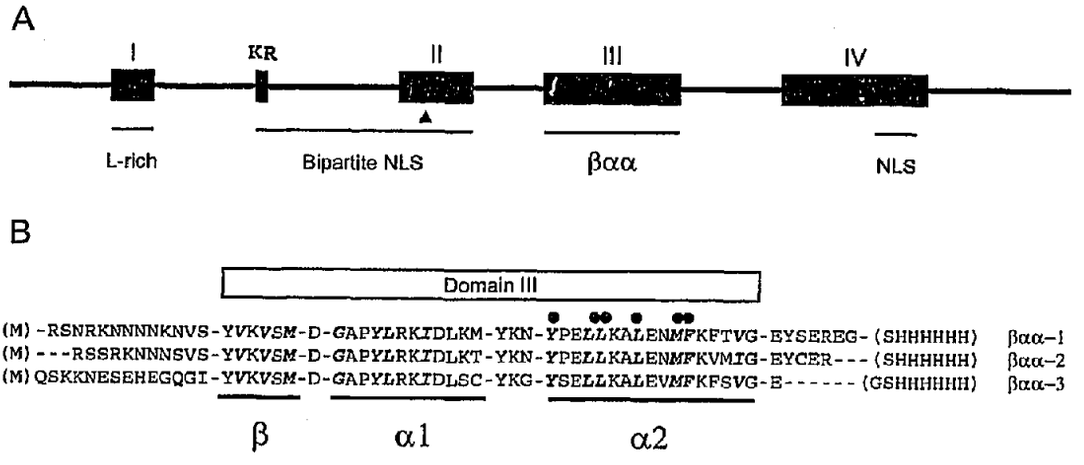

Fig. 1. A: Domain structure of Aux/IAA proteins. Conserved domains I–IV and the invariant basic doublet, KR, are indicated by filled boxes. Functionally identified nuclear localization signals (NLS) [23] and the predicted βαα fold [13] are underlined. A triangle denotes single point mutations in *IAA3* and *IAA17*, which are responsible for the *shy2* [14] and *axr3* [15] phenotype, respectively. B: Primary structure of the recombinant $(His)_6$-tagged peptides derived from conserved domain III. Amino acid residues encoded by the expression vector are given in parentheses. Predicted secondary structural elements are indicated below the alignment. Conserved hydrophobic residues characteristic of the predicted amphipathic βαα fold are in bold and italics. Dots above the alignment denote invariant hydrophobic amino acids at conserved positions, which are predicted to form the hydrophobic surface of amphipathic helix-2.

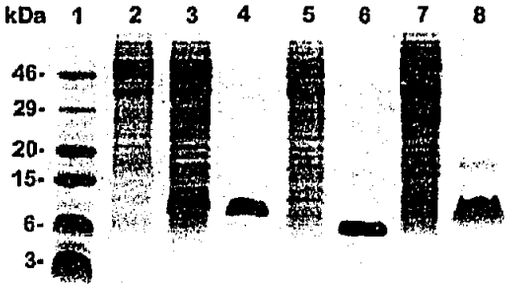

Fig. 2. Expression and purification of $(His)_6$-tagged recombinant domain III peptides of Aux/IAA proteins. Preparation of the bacterial extracts, purification of the $(His)_6$-tagged βαα peptides by Ni-NTA affinity chromatography under denaturing conditions, and electrophoretic separation of the fractions by SDS-PAGE were performed as described in Section 2. Purified protein samples were loaded before the renaturation step and contained 8 M urea. Lane identities: low molecular weight markers (lane 1); total protein extract from host strain, M15[pREP4], containing no expression plasmid (lane 2); total protein extract from induced cells expressing βαα-1 (lane 3), βαα-2 (lane 5), or βαα-3 (lane 7); and Ni-NTA purified recombinant proteins βαα-1 (lane 4), βαα-2 (lane 6), or βαα-3 (lane 8).

lecular mass of about 15 kDa were observed for denatured (8 M urea) βαα-2 and βαα-3 preparations and correspond to the respective dimeric forms of both peptides (see Fig. 2, lane 6 and lane 8). The affinity-purified, denatured recombinant βαα peptide preparations were dialyzed against 10 mM potassium phosphate, pH 8, containing 5 mM EDTA and 100 mM KCl, to allow for renaturation of the βαα peptides. The renatured βαα peptides are soluble at a concentration of up to 2 mg/ml, and were used for the experiments described below.

3.2. Secondary structure analysis by far-UV CD spectroscopy

The structural properties of renatured, $(His)_6$-tagged βαα peptides were evaluated spectroscopically. Far-UV CD spectra for recombinant peptides βαα-1, βαα-2, and βαα-3 were recorded in 10 mM potassium phosphate, pH 8.0, containing 100 mM KCl, at a protein concentration of 50 μg/ml (about 6 μM). As shown in Fig. 3, the CD spectra of all recombinant βαα peptides revealed features characteristic for α-helical secondary structure. The distinctive double minima of large negative ellipticity at 208 nm and 222 nm, and the general shape of the curves, indicates that all βαα peptides tested are folded and have substantial regions of α-helical content [20]. We estimated the α-helical content according to Greenfield and Fasman [20], which ranges from approximately 35% for βαα-2 to 48% for βαα-1. There is no evidence for the presence of β-sheet in either peptide. Next, we examined the ability of the recombinant βαα peptides to fold reversibly after thermal denaturation at 80°C. Upon cooling to room temperature, all βαα peptides fully renature, which is indicated by the production of CD spectra identical to the ones obtained before heat denaturation (data not shown). Thus, although insoluble in *E. coli* and purified under denaturing conditions, reversible folding of the βαα peptides suggests that the observed secondary structure is the authentic fold of expressed recombinant domain III peptides.

3.3. Chemical cross-linking studies

To test our prediction that recombinant βαα peptides dimerize in vitro, we carried out a protein cross-linking analysis with the non-cleavable chemical cross linker BS^3. For the cross-linking experiment, we used conditions that were identical to the spectroscopic CD analysis, that is, renatured, recombinant βαα peptides were studied at a concentration of 50 μg/ml in 10 mM sodium phosphate, pH 8.0, containing 100 mM NaCl. Ribonuclease A, which is a monomeric protein of 13 kDa, was chosen as a negative control for chemical cross-linking. The effect of BS^3 on βαα-1, βαα-2, βαα-3, and ribonuclease A is shown in Fig. 4. When BS^3 is present in the incubation, multiple cross-linked protein bands appear for βαα-1, which is not observed for the control reaction of βαα-1 without BS^3 (compare lane 2 with lane 3). The most prominent cross-linked protein species for βαα-1 corresponds to its dimeric form (approximately 16 kDa), whereas minor protein bands are consistent with multimers of higher order composition (see lane 3). Interestingly, SDS-PAGE of control reactions for renatured βαα-2 and βαα-3 without BS^3 already revealed the presence of multimers, mostly dimers, that are stable under SDS-PAGE conditions (Fig. 4, see lane 4 and lane 6). It should be noted that this phenomenon was consistently observed for βαα-2 and βαα-3 peptides after renaturation of the affinity-purified, denatured peptide preparations. As mentioned above, SDS-PAGE of denatured (8 M urea) βαα-2 and βαα-3 peptides indicated formation of stable

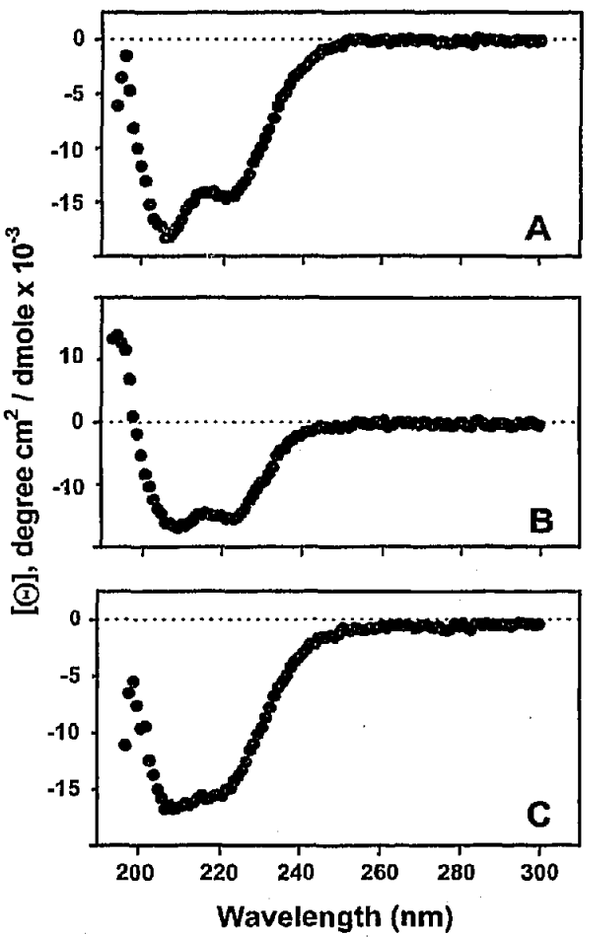

Fig. 3. Far-UV circular dichroism spectra of Ni-NTA purified, renatured $(His)_6$-tagged βαα peptides. The CD spectra were obtained for βαα-1 (A), βαα-2 (B), and βαα-3 (C) as described in Section 2.

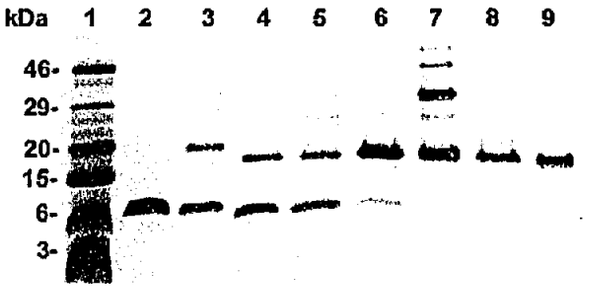

Fig. 4. Chemical protein cross-linking of recombinant βαα peptides and ribonuclease A. Ni-NTA purified, renatured, $(His)_6$-tagged βαα-1 (lanes 2 and 3), $(His)_6$-tagged βαα-2 (lanes 4 and 5), $(His)_6$-tagged βαα-3 (lanes 6 and 7), and ribonuclease A (lanes 8 and 9) were incubated in the absence (lanes 2, 4, 6 and 8) or presence (lanes 3, 5, 7 and 9) of BS^3 as described in Section 2. After termination of the reactions, the proteins were separated on a SDS-PAGE gel, and stained with Coomassie blue. Low molecular weight markers were run in lane 1.

dimers (compare lanes 6 and 8 in Fig. 2 with lanes 4 and 6 in Fig. 4 for βαα-2 and βαα-3, respectively). Cross-linking reactions with BS^3 significantly increased the amount of oligomeric forms of βαα-2 and βαα-3, in particular those corresponding to the respective tetramers (Fig. 4, compare lane 4 with 5, and lane 6 with 7). As expected for the negative cross-linking control, ribonuclease A does not form any oligomeric structures in the presence of BS^3, which indicates the specificity of the cross-linking reaction for the βαα peptides (Fig. 4, compare lane 8 with 9).

Recombinant peptides βαα-1 and βαα-2 differ detectably in size (see Fig. 2, lanes 4 and 6). Therefore, we attempted to test by cross-linking analysis if both βαα peptides interact in vitro. Equal amounts of βαα-1 and βαα-2 were mixed (6 μM each) and preincubated before the addition of BS^3. Separation of the reaction mixture by SDS-PAGE revealed the appearance of three protein bands in the 15 kDa region (Fig. 5, lane 2). The upper and the lower band of the triplet correspond to the homodimer of βαα-1 and βαα-2, respectively, whereas the additional, central band is interpreted to represent the cross-linked βαα-1/βαα-2 heterodimer (Fig. 5, compare lane 2 with lanes 4 and 6).

4. Discussion

Emerging genetic and molecular evidence supports the hypothesis that Aux/IAA proteins play a central role in auxin signaling as transcriptional regulators of middle and late gene expression [9–15,21,22]. However, our knowledge about the structure and the biochemical functions of Aux/IAA proteins and their conserved domains is still in its infancy. Previous studies focusing on Aux/IAA protein function have revealed that *Aux/IAA* genes encode short-lived nuclear proteins [13]. Functional nuclear localization signals have been identified in some Aux/IAA proteins and are part of conserved domain II and domain IV [23]. Domain I is the core of a N-terminal, leucine-rich region and is proposed to function in protein-protein interactions [11]. Domain III contains a predicted βαα motif that is similar to the β-ribbon DNA recognition motif of prokaryotic repressor proteins of the Arc family [11,13]. Based on structural similarities of both motifs, we have previously proposed that the predicted plant βαα fold be involved in dimerization of Aux/IAA proteins [13]. Recently, it has been demonstrated that Aux/IAA proteins homo- and heterodimerize in vitro and in vivo [12]. Moreover, Aux/IAA proteins interact with members of the ARF protein family of transcription factors that contain C-terminal, Aux/IAA-like domains III and IV [9,12]. Using a yeast two-hybrid system, deletion analysis of the PS-IAA4 protein from pea revealed that the C-terminus of PS-IAA4, containing domains III and IV, is necessary and sufficient for dimerization [12]. However, due to the instability of some of the tested PS-IAA4 deletion proteins in yeast, the in vivo data were inconclusive with respect to the role of the predicted βαα fold of domain III in protein dimerization [12].

In this report, we describe a biochemical analysis of the predicted βαα fold of Aux/IAA proteins. We have used CD spectroscopy to probe its secondary structure content, and chemical cross-linking experiments to test its ability to dimerize in vitro. To allow for a comparative analysis, we have expressed and purified $(His)_6$-tagged peptides corresponding to domain III of three *Arabidopsis* proteins, IAA1, IAA2, and IAA3. As previously predicted by several secondary structure algorithms and by helical wheel analysis, the region centered on domain III is likely to adopt an amphipathic βαα fold. In particular, the five invariant hydrophobic amino acid residues at conserved positions in domain III are consistently predicted with high probability to fold into the amphipathic, second helix of the βαα motif [13]. The CD spectra obtained for the recombinant βαα peptides tested indicate significant α-helical structure content of domain III (Fig. 3). Reversibility of protein folding after thermal denaturation implies that domain III of Aux/IAA polypeptides folds autonomously and represents a stable protein fold. Furthermore, chemical cross-linking analysis demonstrates that the βαα peptides form homodimers and heterodimers in vitro (Figs. 5 and 6). These results are in agreement with our proposition that domain III of Aux/IAA proteins is structurally and functionally related to the βαα fold of Arc-like, prokaryotic transcriptional repressors. However, two observations suggest unique properties of the Aux/IAA βαα domain. First, unlike Arc-like polypeptides, oligomers of the βαα domain of Aux/IAA proteins are unusually stable. In the absence of chemical cross-linker, homodimers of renatured βαα peptides are consistently detected by SDS-PAGE, which demonstrates formation of dimers that are sufficiently stable to withstand the denaturing

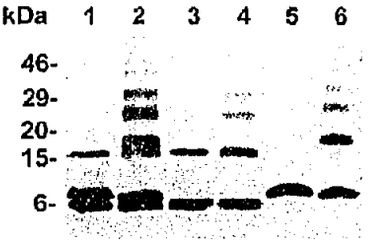

Fig. 5. Chemical cross-linking of βαα-1/βαα-2 complexes. Equal amounts of Ni-NTA purified, renatured, $(His)_6$-tagged βαα-1 and $(His)_6$-tagged βαα-2 (2.5 μg each) were mixed (see Section 2) and preincubated at room temperature for 10 min prior to the control (lane 1) and cross-linking incubation (lane 2). Controls and cross-linking reactions for the individual peptides, $(His)_6$-tagged βαα-1 (lanes 3 and 4) and $(His)_6$-tagged βαα-2 (lanes 5 and 6) were performed as described in Fig. 4. After termination of the reactions, the proteins were separated on a SDS-PAGE gel, and stained with Coomassie blue.

conditions of SDS-PAGE (Fig. 4). Second, the recombinant βαα peptides tend to aggregate in solution at a protein concentration higher than 20 μM (data not shown). Conversely, the dimeric Arc and tetrameric Mnt proteins do not form soluble aggregates at concentrations ranging from 2 to 200 μM [24]. It may be argued that the addition of the C-terminal $(His)_6$ tag is responsible for the association behavior of the expressed βαα peptides. However, C-terminally $(His)_6$-tagged recombinant Arc repressor protein, which is of similar size (64 amino acids) and predicted secondary structure as the $(His)_6$-tagged βαα peptides, has virtually identical association properties and CD spectra as wild type Arc protein [25]. Thus, it is unlikely that the C-terminal $(His)_6$ tag significantly affects the biochemical properties of the βαα peptides characterized in our study.

It is evident from the crystal structures of Arc and MetJ that the amphipathic helix-2 of the prokaryotic βαα fold mediates dimerization [16]. Accordingly, helix-2 of the Aux/IAA βαα domain is likely to mediate oligomerization of the βαα fold. Furthermore, the striking amphipathic nature of helix-2 of Aux/IAA proteins (Fig. 1B), which is less prominent for helix-2 of Arc-like proteins (see [13]), likely facilitates formation of stable oligomers of βαα peptides in solution. An important role of helix-2 in Aux/IAA protein dimerization is also suggested by the truncated βαα domain found in IAA25. Although domain III of IAA25 lacks the predicted β-sheet motif and a substantial region of helix-1, IAA25 still interacts with IAA1 in vivo, presumably via the remaining helix-2 [12]. This observation and our experimental data suggest that domain III plays a critical role in homo- and heterodimerizations of Aux/IAA and ARF proteins, which are proposed to govern differential expression patterns of secondary genes in response to auxin. Biochemical and biophysical studies of mutant variants that contain single amino acid substitutions in domain III will allow for evaluating the contribution of individual residues to protein-protein interactions and to the function of the βαα domain in Aux/IAA and ARF proteins.

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